Amendments to Drawings:

The attached replacement sheets and annotated sheets include changes to sheets 1 through 5 of the original drawings, and changes to Figs. 3 and 4a. The figure numbers on sheets 1 through 5 have been amended to translate the original German text into English text.

On sheet 3, in Fig. 3, the German term "Primer-komb." has been translated into English as "Primer-comb."

On sheet 4, in Fig. 4a, several terms have been translated into English from German.

Attachments: 5 Replacement Sheets

5 Annotated Sheet Showing Changes

REMARKS

Reconsideration of this application and withdrawal of the rejections set forth in the non-final Office Action mailed July 6, 2010, is requested in view of this amendment and the following remarks. Claims 29, 30 and 70-95 were pending and at issue prior to this amendment. By this amendment, claims 29 and 30 have been amended. In addition, sheets 1-5 of the drawings have been amended, as described above. The claims and drawings as amended are fully supported by the original specification as filed, and no new matter has been added.

I. Objections to the Drawings

The Examiner objected to the drawings on the basis that the drawings have non-English text. The drawings have been amended as described above, and shown on the accompanying annotated drawings and replacement drawings. The amendments have replaced the original German text with English translations.

Applicants submit the basis for the Examiner's rejections has been removed, and that the objection to the drawings should be withdrawn.

II. Claim Objections

The Examiner objected to claims 29 and 87 as having recitations in improper Markush form. Applicants' have amended claims 29 and 87 into proper Markush format by replacing the term "comprising" with the term "consisting of."

Accordingly, Applicants submit that this objection to the claims should be withdrawn.

III. Rejections under 35 U.S.C. Section 112, second paragraph

Claims 30, 71, 73, 76, 79 82 and 85 stand rejected under 35 U.S.C. Section 112, second paragraph, as being indefinite. Specifically, the phrase "for example" in claim 30 is asserted to render the claim indefinite. Claim 30 has been amended to remove the phrase "for example" thereby removing the basis for this rejection. Claims 71, 73, 76, 79 82 and 85 were rejected hereunder solely on the basis of their dependence on claim 30. Therefore, the amendment to claim 30 also removes the basis of the rejection of claims 71, 73, 76, 79 82 and 85, as well.

Therefore, Applicants submit that this rejection should be withdrawn.

IV. Rejections under 35 U.S.C. Section 112, first paragraph

Claims 30, 71, 73, 76, 79 82 and 85 stand rejected under 35 U.S.C. Section 112, second paragraph, as based on a disclosure which is not enabling. The Examiner asserts that the recitation of the dimensions stated in the recommendations of the SBS (Society of Biomolecular Screening) is not enabled by the disclosure and that the limitation amounts to an incorporation by reference. Applicants disagree with this characterization of the SBS standards recited in claim 30. The SBS standards are standards set by ANSI (American National Standards Institutes). Those of ordinary skill in the relevant art of the invention will understand the term as set forth in claim 30, such that the claim is not based on a disclosure which is not enabling. The use of the reference to the SBS standards is clearly enabled, and one of ordinary skill in the art plainly is able to immediately and easily access, determine and understand the requirements of the SBS standards.

Moreover, the SBS standards are not required to be recited in the specification as essential material. The recitation of the SBS standards is similar to the recitation of an IEEE

standard (see U.S. Patent No. 7,865,754, which includes IEEE references in the claims), or the recitation of the term "internet protocol" in a claim. Such recitations do NOT require that the entire specification of the IEEE or a full explanation of "internet protocol" be included in the specification. Those of ordinary skill in the art understand these terms, and their requirements.

Nevertheless, Applicants have amended claim 30 to remove the reference to the SBS standards, without acquiescing to the rejection, and explicitly reserving the right to pursue claims having the same or similar limitations as claim 30 prior to the amendment. Claims 71, 73, 76, 79 82 and 85 were rejected hereunder solely based on their dependence on claim 30.

Therefore, Applicants submit that this rejection should also be withdrawn.

V. Obviousness Rejections under 35 U.S.C. Section 103

Claims 29, 30, 70-80, 94 and 95 stand rejected under 35 U.S.C. section 103(a) as being obvious over Thunnissen et al. (WO 03/087829), in view of GenBank GI:60955 [online] July 6, 1989 [retrieved on July 3, 2010] retrieved from:

http://www.ncbi.nlm.gov/sviewer/viewer.fcgi?val=60955&sat=OLDID&satkey=34726

(hereafter, "GenBank GI:60955).

Claims 81-83 stand rejected under 35 U.S.C. section 103(a) as being obvious over Thunnissen et al., in view of GenBank GI:60955, and further in view of Amundson et al. (WO 00/50643).

Claims 84-86 stand rejected under 35 U.S.C. section 103(a) as being obvious over Thunnissen et al., in view of GenBank GI:60955 and Amundson et al., and further in view of Fodor et al. (U.S. Patent Appn. Publ. No. 2003/0186296).

Claims 87 and 88 stand rejected under 35 U.S.C. section 103(a) as being obvious over

Thunnissen et al., in view of GenBank GI:60955, Amundson et al. and Fodor et al., and further in view of Walkerpeach et al. (U.S. Patent Appn. Publ. No. 2001/0006800).

Claims 89-91 stand rejected under 35 U.S.C. section 103(a) as being obvious over Thunnissen et al., in view of GenBank GI:60955, and further in view of Sabath et al. (U.S. Patent Appn. Publ. No.2003/0175761).

Claim 92 stands rejected under 35 U.S.C. section 103(a) as being obvious over Thunnissen et al., in view of GenBank GI:60955, and further in view of Neefe et al. (U.S. Patent Appn. Publ. No.2003/0170268).

Claim 93 stands rejected under 35 U.S.C. section 103(a) as being obvious over Thunnissen et al., in view of GenBank GI:60955 and Neefe et al., and further in view of Maas et al. (PNAS 96:8895-8900, August 1999).

Applicant respectfully submits that all of the obviousness rejections should be withdrawn because neither Thunnissen et al., nor any of the other cited prior art, disclose, suggest or otherwise render obvious the claimed nucleotide array for detecting and/or identify nucleotide array for detecting and/or identifying the genotype of a human papilloma virus contained in a biological sample comprising a solid carrier having a surface and at least one first oligonucleotide or nucleic acid molecule bound to the carrier surface that is suitable for use as a probe for testing the HPV gene E1 or a portion thereof to detect and/or identify a genital human HPV genotype selected from the group consisting of:

a) HPV genotype-specific oligonucleotides having the nucleotide sequences recited in SEQ ID nos. 8 to 135,

b) oligonucleotides that have a nucleotide sequence that is mutated relative to one of the

oligonucleotides of a), namely, a deletion or addition of 1 to 10 nucleotides or a substitution of 1 to 3 nucleotides in one of the nucleotide sequences recited in a),

c) oligonucleotides that have a nucleotide sequence that is complementary over its entire length to the nucleotide sequence of an oligonucleotide of a) or b) ,

d) nucleic acid molecules comprising at least one region that has one of the nucleotide sequences recited in a) to c) and one or more additional regions having a total length of at least one nucleotide, and

e) mixtures of the oligonucleotides of a) to c) and/or of the nucleic acid molecules of d).

While the Supreme Court in *KSR International Co. v. Teleflex Inc.* ("KSR")[1] held that there are no rigid rules for determining obviousness, the Court reaffirmed that "rejections on obviousness cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinnings to support the legal conclusion of obviousness."[2] As reiterated by the Supreme Court in KSR, the framework for the objective analysis for determining obviousness under 35 U.S.C. §103 is stated in *Graham* v. *John Deere Co.*[3] Obviousness is a question of law based on underlying factual inquiries. The factual inquiries enunciated by the Court are as follows:

(1) Determining the scope and content of the prior art;

(2) Ascertaining the differences between the claimed invention and the prior art; and

(3) Resolving the level of ordinary skill in the pertinent art; and

(4) Considering objective evidence (secondary considerations) indicating obviousness or

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1 550 U.S. ___, ___ (2007)
2 550 U.S. ___, ___ (2007)
3 550 U.S. ___, ___ (2007)

non-obviousness.

Although the Court in KSR rejected rigid preventative rules in making an obviousness inquiry, the Court reaffirmed that there must be some articulated rationale in order to modify or combine the teachings of multiple references in making an obviousness rejection.[4] Furthermore, the combined teachings of the prior art must at least result in the Applicant's claimed invention.

Therefore, to establish a *prima facie* case of obviousness, the Examiner must establish three basic criteria: first, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; second, there must be a reasonable expectation of success; and finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. Moreover, the teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). It is improper to pick and choose among features and elements found in the prior art references in a hindsight reconstruction of Applicants' claimed invention. *See e.g., In re Jones*, 958 F.2d 347, 350-51 (Fed. Cir. 1992); *In re Fritch*, 919 F.2d 720 (Fed Cir. 1990) ("It is impermissible to use the claimed invention as an instruction manual or 'template' to piece together the teachings of the prior art so that the claimed invention is rendered obvious"). Furthermore, it is improper to combine the teachings of references where the references teach away from the asserted combination. MPEP § 2145.

Moreover, unexpected results produced by the claimed invention may rebut a *prima facie*

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[4] 550 U.S. ___, ___ (2007)

case of obviousness. MPEP § 716.02 *et seq.* "A greater than expected result is an evidentiary factor pertinent to the legal conclusion of obviousness ... of the claims at issue." MPEP § 716.02 (a). Applicant submits that the Examiner's obviousness rejection cannot be sustained consistent with these well-established requirements for obviousness.

The present invention, as recited in claim 29, includes probes for the HPV E1 gene which are non-obvious over the disclosure of different probes disclosed by Thunnissen et al. because the probes of claim 29 produce unexpectedly advantageous results. Those of ordinary skill in the art would not reasonably anticipate that Applicant's claimed probes would have the advantages described below.

Thunnissen et al. discloses a microarray for the detection of human papilloma viruses, wherein a combination of oligonucleotides is used for the amplification of parts of the respective HPV E1 gene. Table 1 of Thunnissen et al. describes the different probes (SEQ 10 No. 24 to 59) and amplification primers (SEQ 10 No. 1 to 23). On page 4, first paragraph, Thunnissen et al. states that the 3'-end of the HPV E1 gene is preferred for the amplification, in particular a region lying between 29 and 188 nucleotides from the 3'-terminus of the E1 gene. Also, Figure 1 of Thunnissen et al. shows that the disclosed probes and amplification primers, visualized by the location of the CWZ-primer, are located near the 3'-end of the E1 gene.

The technical problem underlying the present invention is therefore the provision of improved means to diagnose HPV infections.

The present invention solves this technical problem by the provision of a nucleotide array, wherein the employed probes for the E1 gene are not localized at the 3'-end of the E1 gene as in Thunnissen et al., but are instead localized further in the 5'-direction of the E1 gene. In

fact, the probes according to the present invention detect sequences in a region of the E1 gene which is 770 to 406 base pairs (bp) apart from the 3'-end depending on HPV type, since the PCR product begins at positions 770 to 752 and ends at positions 424 to 406, calculated from the 3'-end of the respective E1 genes. This results from the localization of the amplification primers of the present invention which cover a region from 2056 to 2402 (according to HPV16 genome; for HPV6: 2027 to 2373), and therefore are much further away from the 3'-terminus of the E1 gene than the amplification primers of Thunnissen et al. Surprisingly, when used in a diagnostic method, the specific localization of the probes and amplification primers according to the present invention provides the ability to obtain far more reliable results than the probes disclosed in Thunnissen et al.

Without being bound by the theory underlying the improved results, this advantage might be due to the situation in which a likely deletion of the E2 gene during the integration of the HPV genome into the host cell's genome also leads to the deletion of parts of the 3'-end of the E1 gene, wherein an attempted detection of those 3'-sequences would accordingly be unsuccessful. In fact, during the development of a cervix carcinoma, the HPV genome is often integrated into the genome of the host cell. During this integration event, parts of the HPV genome are usually lost, and only the E6 and E7 genes, which are the most oncogenic genes, are always retained. In contrast, the E2 gene is often deleted which is shown by the publication of John Doorbar (2005), in particular page S13, left column (attached hereto as Annex 2). It is possible that a deletion of the E2 gene even increases the risk of the development of a cervix carcinoma. An integration event during which the E2 gene is lost and which accordingly bears a high risk for the development of cancer very likely also leads to a deletion of the 3'-end of the

neighboring E1 gene. This is due to the fact that the 5'-end, i.e. the beginning, of the E2 gene overlaps with the 3'-terminus, i.e. the end, of the preceding E1 gene. This can also be seen in Figure 1 of Thunnissen et al. In exact terms, the E1 gene of HPV 16, for example, begins at position 865 and ends at 2814 and the E2 gene already begins at position 2756. In the genome of HPV 6 the E1 gene begins at 883 and ends at 2782, whereas the E2 gene already begins at 2724. As shown in Figure 1 of Thunnissen et al., Thunnissen et al. teaches that it is this overlapping region in which the amplification primers and probes are located to detect a HPV infection.

Since a deletion event of the E2 gene thus includes sequences of the 3'-end of the E1 gene, the position of the reverse primer, that is the 3'-primer, of the PCR product to be detected by the probes is crucial. A detailed analysis of the primers for HPV 6 of Thunnissen et al. shows that the reverse primers 21, 6, 2 and 4 are located at positions 2660, 2742, 2937 and 2991. In contrast, the reverse primer of the present invention which is located furthest towards the 3'-end of the E1 gene is located at position 2373 of HPV 6 and therefore almost 300 nucleotides further in the secure 5'-direction of the gene. The same holds true for the amplification primers for HPV 16. Here, the reverse primers of Thunnissen et al., namely SEQ 10 No.8, 11, 13, 17, 18, 19,22 and 23 are located at positions 2677 (SEQ 10 17), 2689 (SEQ IDs 18, 19,22,23),2755 (SEQ 108) and 2919 (SEQ 10 11, 13). The reverse primer of the present invention which is located furthest in 3'-direction of HPV 16 ends at position 2402, so that again more than 200 nucleotides lie between the respective reverse primers.

The enclosed Annex 1 shows all of the probe positions according to Thunnissen et al. (SEQ 10 No. 24 to 59) in relation to the position of the reverse primer of the present invention which is located nearest to the 3'-end of the E1 gene (Loma rev, SEQ ID No 7 of the present

invention). Also, the number of nucleotides between the probe according to Thunnissen et al., and the position of this reverse primer is given. It becomes apparent from Table 1 of Annex 1 that there is a gap of at least 248 nucleotides, depending on HPV type, between the reverse primer according to the present invention and the probes according to Thunnissen et al. which are located furthest in the 5'-direction. As can also be seen from Table 1, 19 of the probes according to Thunnissen et al. actually lie behind the stopcodon of the E1 gene, i.e. completely within the E2 gene of the respective HPV type. This clearly confirms the above-discussed effect.

In the event of a deletion of the E2 gene, which is very likely during an integration event, the method according to Thunnissen et al. would, therefore, not provide reliable results. In contrast, the use of the oligonucleotide arrays according to the present invention would still produce reliable results in case of a deletion of the E2 gene, since the region overlapping with the E1 gene does not extend to the region detected by the probes according to the present invention. The use of the oligonucleotide arrays according to the present invention, therefore, also allows the detection of persistent HPV infections in which the risk of cancer development is very high.

Therefore, Thunnissen et al. does not render obvious the probes of Applicants' claimed invention. Furthermore, none of the other prior art cited by the Examiner would teach, suggest or motivate one of ordinary skill in the art to modify the teachings of Thunnissen et al. to produce the specific he probes of Applicants' claimed invention.

Accordingly, independent claim 29 is not obvious over Thunnissen et al., in view of the other cited prior art. Claims 30 and 70-95 depend from claim 29, or an intervening claim, and are not obvious over Thunnissen et al., in view of the other cited prior art, for at least the same

reasons applicable to claim 29. Thus, all of the obviousness rejections should be withdrawn.

Conclusion

Any claim amendments which are not specifically discussed in the above remarks are not made for reasons of patentability, do not affect the scope of the claims, and it is respectfully submitted that the claims satisfy the statutory requirements for patentability without the entry of such amendments. These amendments have only been made to increase claim readability, to improve grammar, or to reduce the time and effort required of those in the art to clearly understand the scope of the claim language.

In view of the foregoing remarks, Applicant respectfully submits that all of the Examiner's rejections have been overcome. Accordingly, allowance is earnestly solicited. If the Examiner feels that a telephone interview could expedite resolution of any remaining issues, the Examiner is encouraged to contact Applicant's undersigned representative at the phone number listed below.

Respectfully submitted,

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ANNEX 1

Annex 1

Table 1 provides the 5'-positions of the probe sequences (SEQ ID No. 24 to 59) disclosed in Thunnissen et al. (WO 03/087829) within the genome of the specific HPV types. In comparison thereto the positions of the Loma-PCR-product (furthest downstream position of the Loma reverse primer) of the present invention and the position of the HPV type-specific E1 gene as well as the startcodon of the E2 gene is stated.

Remarks:

All specific positions in one row relate to the HPV type given in the first column.

Footnotes:

1 SEQ ID-Numbers of the probes according to Thunnissen et al.

2 **bold**: whole probe lies after stopcodon of the E1 gene (HPV 6, 7, 11, 13, 16, 18, 31, 32, 33, 35, 40, 42, 44, 45, 52, 55, 58, 59, 67)
italic: probe lies on stopcodon of the E1 gene (HPV 39, 70, 85)
underlined: probe lies on the startcodon of the E2 gene (HPV 2, 3, 10, 28, 57)

Since the E1 and E2 genes overlap all probes marked red and yellow lie completely within the E2 gene and probes marked blue lie at least partially within the E2 gene.

3 the furthest downstream position is given

Table 1

SEQ ID[1]	Type	5'-position of the probe[2]	Position Loma rev[3]	Nucleotides between probe and Loma rev	Type-specific position of E1 gene	Type-specific position of E2 startcodon	comment
24	HPV2	2668	2331	337	812-2743	2685	
25	HPV3	2710	2373	337	806-2785	2727	
26	HPV6	**2904**	2373	531	883-2782	2724	
27	HPV7	**2935**	2400	535	868-2808	2750	
28	HPV10	2786	2424	362	791-2836	2778	
29	HPV11	**2903**	2372	531	832-2781	2723	
30	HPV13	**2907**	2374	533	843-2783	2725	
31	HPV16	**2825**	2401	424	865-2814	2756	
32	HPV18	**2972**	2472	500	914-2887	2817	
33	HPV28	2711	2365	346	788-2776	2718	
34	HPV31	**2758**	2339	419	861-2751	2693	
35	HPV32	**2899**	2366	533	850-2778	2720	

36	HPV33	2870	2395	475	879-2813	2749	
37	HPV34	2630	2382	248	851-2794	2733	
38	HPV35	2761	2345	416	868-2760	2693	
39	HPV39	2860	2456	404	928-2871	2798	
40	HPV40	2938	2405	533	868-2811	2753	
41	HPV42	2881	2348	533	724-2760	2672	
42	HPV44	2803	2354	449	832-2763	2705	
43	HPV45	2930	2430	500	914-2845	2769	
44	HPV52	2864	2389	475	864-2807	2743	
45	HPV54	2633	2314	319	828-2729	2671	
46	HPV55	2826	2354	472	829-2763	2705	
47	HPV56	2686	2392	294	No official definition	2918	
48	HPV57	2666	2329	337	810-2741	2683	
49	HPV58	2874	2399	475	883-2817	2753	
50	HPV59	2819	2391	428	872-2806	2736	
51	HPV61	2667	2345	322	811-2769	2705	
52	HPV67	2842	2367	475	876-2786	2721	
53	HPV69	2674	2381	293	886-2790	2732	
54	HPV70	2875	2471	404	928-2886	2813	
55	HPV72	2687	2365	322	832-2783	2719	(E1a and E1b)
56	HPV73	2638	2390	248	850-2802	2741	
57	HPV82	2678	2395	283	876-2804	2746	
58	HPV83	2571	2252	319	718-2673	2615	
59	HPV85	2851	2445	406	920-2866	2799	

ANNEX 2

ELSEVIER

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Review

The papillomavirus life cycle

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Abstract

Papillomaviruses infect epithelial cells, and depend on epithelial differentiation for completion of their life cycle. The expression of viral gene products is closely regulated as the infected basal cell migrates towards the epithelial surface. Expression of E6 and E7 in the lower epithelial layers drives cells into S-phase, which creates an environment that is conducive for viral genome replication and cell proliferation. Genome amplification, which is necessary for the production of infectious virions, is prevented until the levels of viral replication proteins rise, and depends on the co-expression of several viral proteins. Virus capsid proteins are expressed in cells that also express E4 as the infected cell enters the upper epithelial layers. The timing of these events varies depending on the infecting papillomavirus, and in the case of the high-risk human papillomaviruses (HPVs), on the severity of neoplasia. Viruses that are evolutionarily related, such as HPV1 and canine oral papillomavirus (COPV), generally organize their productive cycle in a similar way, despite infecting different hosts and epithelial sites. In some instances, such as following HPV16 infection of the cervix or cottontail rabbit papillomavirus (CRPV) infection of domestic rabbits, papillomaviruses can undergo abortive infections in which the productive cycle of the virus is not completed. As with other DNA tumour viruses, such abortive infections can predispose to cancer.

Keywords: Human papillomavirus; Productive infection; Latency; Warts; Papillomas; Cervical cancer

Contents

Abbreviations: HPV, human papillomavirus; BPV, bovine papillomavirus; COPV, canine oral papillomavirus; CRPV, cottontail rabbit papillomavirus; PML, promyelocytic leukaemia; NMSC, non-melanoma skin cancer

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1. Diversity amongst human papillomaviruses

Papillomaviruses are a diverse group of viruses that have been found in more than 20 different mammalian species, as well as in birds and reptiles. Because of their medical importance, the human papillomaviruses (HPV) have been most extensively studied, and more than 100 different types have now been identified (Bernard, 2005). Although papillomavirus classification is based on nucleotide sequence homology, the differences between evolutionary groups are reflected to some extent, in the differences that exist in the biology of the different viruses. Genitally transmitted HPV types are contained within supergroup A (also known as Alpha papillomaviruses) (de Villiers et al., 2004; Myers et al., 1994) and viruses from this group, such as HPV6 and 11, are major sexually transmitted pathogens that are thought to affect around 1% of the sexually active population (Brentjens et al., 2002). These viruses can also infect oral sites where they are generally associated with benign papillomas. By contrast, the high-risk viruses from supergroup A, such as HPV16 and 18, cause mucosal lesions that can progress in some individuals to high-grade neoplasia and cancer (Bosch et al., 2002; Walboomers et al., 1999). Although viruses from supergroup A also include members whose primary tropism is for cutaneous sites, such as HPV2 or HPV10, these viruses share life cycle features that do not extend to papillomaviruses from other evolutionary groups (Middleton et al., 2003; Peh et al., 2002). HPV2 and closely related supergroup A papillomaviruses are the primary cause of common warts.

The second major group of human papillomaviruses are contained within supergroup B. Viruses from the B1 subgroup such as HPV5 (also known as Beta papillomaviruses) (de Villiers et al., 2004; Myers et al., 1994) cause inapparent or latent infections in the general population, but can become a problem in immuno-suppressed individuals and in individuals who have an inherited defect (Ramoz et al., 2002) which renders them susceptible to infection by papillomaviruses from the B1 supergroup. Such patients can develop skin cancers at the site of HPV infection, and it is thought that B1 viruses may be involved in the development of non-melanoma skin cancer (NMSC) in the general population (Harwood et al., 2004). By contrast, viruses from the B2 subgroup such as HPV4 (also known as Gamma papillomaviruses; de Villiers et al., 2004), cause cutaneous warts in the general population that can superficially resemble those caused by supergroup A papillomaviruses such as HPV2.

The remaining group of HPVs are contained within supergroup E (Myers et al., 1994) (also classified as Mu and Nu-papillomaviruses (de Villiers et al., 2004)). Only three human members from this group are known, and all cause cutaneous papillomas in the general population. HPV1 is the most well studied member of this group, and like HPV2 in supergroup A, causes verrucas and palmar warts.

2. Problems in developing a general model of HPV-associated disease

It is apparent from the above overview that different HPVs have evolved to fill different biological niches, and that in some instances, viruses from different evolutionary groups may be able to target the same epithelial site. Despite this apparent heterogeneity amongst HPVs, they all share certain features that allow them to produce infectious virions following infection. All known HPVs are exclusively epitheliotropic, and unlike certain animal papillomavirus types such as bovine papillomavirus type 1 (BPV1) or BPV2, they do not infect or express their gene products in the underlying dermis. Similarly, all produce infectious particles in the upper epithelial layers, although there appear to be differences in the extent of virus synthesis depending on whether transmission is through direct contact (e.g., genital warts), or whether it occurs indirectly (e.g., verrucas) (Middleton et al., 2003; Peh et al., 2002). While we have a basic understanding of how papillomaviruses cause disease, it is becoming apparent that the evolutionary backgrounds of the different viruses, their site of infection, and their mode of transmission must all be considered if the general model is to be applied to particular HPV types. Differences in regulatory sequences and coding potential within the viral genome are likely to underlie the significant differences that are apparent in the biology of different papillomavirus types.

3. Organization of the HPV life cycle

Most work on HPVs has centred on the analysis of the high-risk HPV types and in particular on HPV16, which is the primary cause of cervical cancer. From these studies and from the analysis of related HPV types (including HPV11 and HPV1), a general pattern of viral gene expression has been worked out (Fig. 1) that can, with modification, be applied to human papillomaviruses from other groups.

3.1. Infection and uncoating

Initial infection requires access of infectious particles to cells in the basal layer, which for some HPV types is thought to require a break in the stratified epithelium. Such breaks may not be readily apparent, and may occur under conditions where the skin is exposed to water or is abraded (e.g., swimming pool surfaces), or is subjected to other environments where micro traumas may develop. It has been suggested that for a lesion to be maintained, the virus must infect an epithelial stem cell (Egawa, 2003; Schmitt et al., 1996). In cutaneous skin, such stem cells are abundant within the hair follicle, and for viruses of the B1 supergroup (which are prevalent but which cause inapparent infections), the hair follicles may represent an important site of entry. Several studies have shown that DNA of viruses from the B1 supergroup can be readily amplified by PCR from plucked

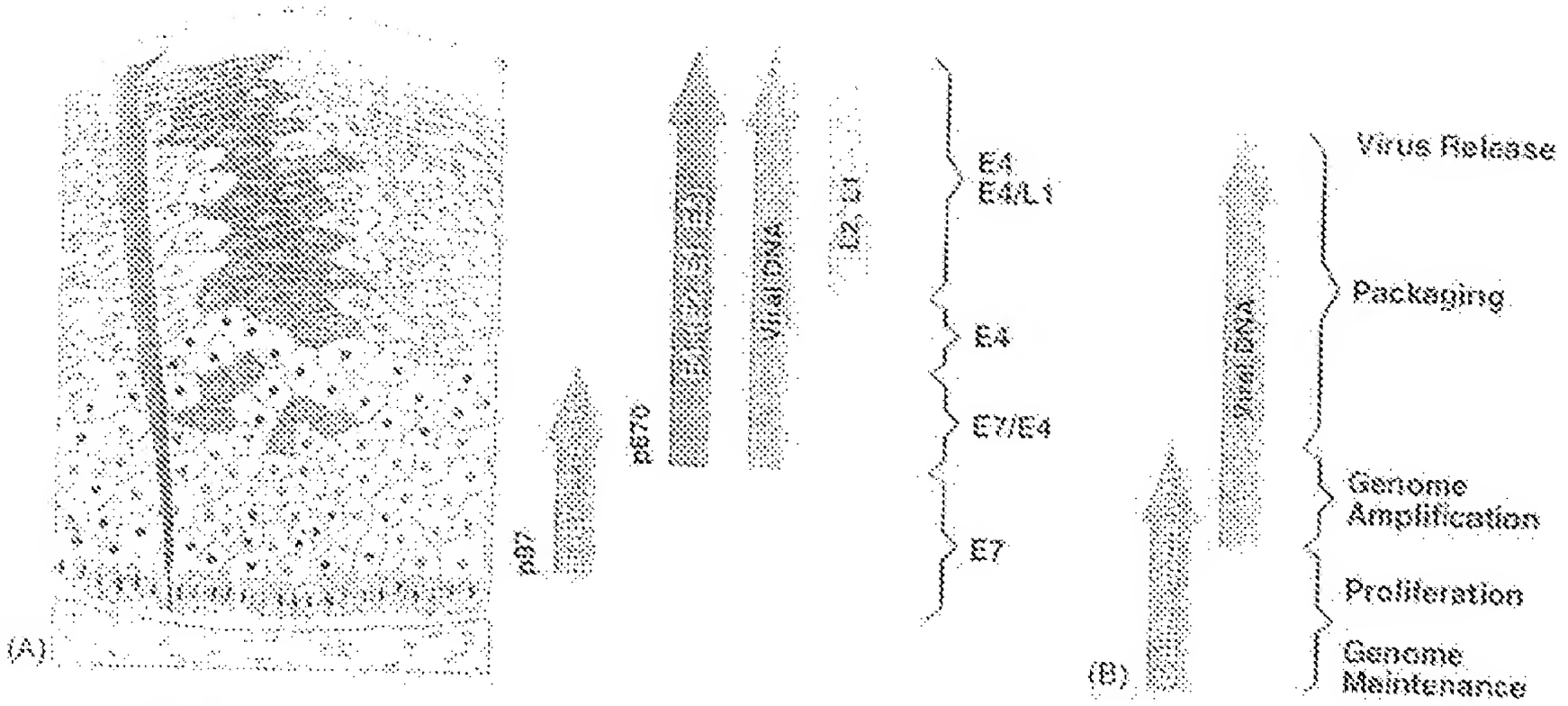

Fig. 1. Life cycle organization during productive infection by HPV types from supergroup A. (A) Diagrammatic representation of the skin to reveal the pattern of HPV16 gene expression as the infected cell migrates towards the epithelial surface. Other supergroup A viruses, such as HPV2 and HPV11 follow a similar pattern. After infection (in this case through a cut), the viral genome is maintained as a low copy number episome. During epithelial differentiation, the p97 promoter directs expression of the E6 and E7 genes necessary for S-phase entry (red). The p670 promoter is up-regulated in the higher epithelial layers, and viral replication proteins (E1, E2, E4, E5) increase in abundance (green), facilitating amplification of viral genomes (blue). Changes in mRNA splicing allow E4 to persist into the upper epithelial layers where the viral capsid proteins (yellow) are found. (B) Cells in the lower epithelial layers are S-phase competent. Viral genome amplification begins in these cells but ceases once the cells lose their ability to express S-phase proteins. Although amplified viral genomes can be detected throughout the upper epithelial layers, cells that are actively supporting genome amplification appear confined to a region where E7 expression coincides with the high-level expression of E4, and probably also, an increase in the abundance of E1 and E2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

hair follicles (Boxman et al., 2001). For high-risk mucosal viruses such as HPV16, the formation of cervical lesions may be facilitated by infection of columnar cells, which can subsequently go on to form the basal layer of the stratified epithelium of the transformation zone. Controversy exists as to the nature of the cell surface receptor that allows initial attachment of the virus to the cell, although most studies have suggested a dependence on the presence of heparin sulphate (Giroglou et al., 2001; Joyce et al., 1999). Recent work has suggested that the internalization of bound virions is a slow process with a half-life of hours rather than minutes, and that occurs through the endocytosis of clathrin coated vesicles (Culp and Christensen, 2004; Day et al., 2003; Selinka et al., 2002). Papillomavirus uncoating may be facilitated by the disruption of intracapsomeric disulphide bonds in the reducing environment of the cell (Li et al., 1998) allowing viral DNA to be transported into the nucleus.

3.2. Genome maintenance

Following infection and uncoating, it is thought that the virus maintains its genome as a low copy number episome in the basal cells of the epithelium. The pattern of viral gene expression in these cells is not well defined, but it is generally thought that the viral E1 and E2 proteins are expressed in order to maintain the viral DNA as an episome (Wilson et al., 2002) and to facilitate the correct segregation of genomes during cell division (You et al., 2004). Failure to express the full length E1 protein in the context of the HPV31 genome, prevents episomal maintenance, and in cultured epithelial cells leads to the integration of viral genomes into the host cell chromosome (Frattini et al., 1996). Whether the viral 'transforming' proteins (E6 and E7) are also expressed in cells of the basal layer is not certain (Crum et al., 1988), although it appears that initial infection is followed by a proliferative-phase that results in an increase in the number of basal cells harbouring viral episomes. The number of viral genomes, and the pattern of viral gene expression in cell lines derived from low-grade cervical lesions appears to reflect those found in the basal layer of naturally-occurring lesions. It has been suggested that the viral genome is maintained in the basal layer at around 10–200 copies per cell, and that viral early proteins (E6, E7, E1 and E2) are expressed at low level (De Geest et al., 1993; Stanley et al., 1989). The contribution of E6 and E7 to basal cell proliferation during in vivo infection is currently uncertain, and it has been suggested that expression of E1 (and possibly also E2) may be sufficient for the basal maintenance of viral episomes (Zhang et al., 1999).

3.3. Proliferative-phase

In uninfected epithelium, basal cells exit the cell cycle soon after migrating into the suprabasal cell layers and undergo a process of terminal differentiation. Changes include the physical cross-linking of keratin intermediate filaments, the formation of cornified envelopes, and the secretion of lipids, which together allow the epithelial surface to form a physical barrier against the environment (Madison, 2003). During papillomavirus infection, E7 (and presumably also

E6) is expressed in these cells, the restraint on cell cycle progression is abolished and normal terminal differentiation is retarded (Sherman et al., 1997). E6 and E7 are thought to work together to achieve these effects, and in lesions caused by high-risk HPV types (such as HPV16), the two proteins are expressed from a bicistronic mRNA (Stacey et al., 2000) expressed from the viral early promoter (p97). Both E6 and E7 have functions that stimulate cell cycle progression and both can associate with regulators of the cell cycle (Munger et al., 2001). The association of E7 with members of the pocket protein family such as pRb is well characterized. pRb is a negative regulator of the cell cycle that normally prevents S-phase entry by associating with the E2F family of transcription factors. E7 binding to pRb displaces E2F, irrespective of the presence of external growth factors, and leads to the expression of proteins necessary for DNA replication. E7 can also associate with other proteins involved in cell proliferation, including histone deacetylases (Longworth and Laimins, 2004), components of the AP-1 transcription complex (Antinore et al., 1996), and the cyclin-dependent kinase inhibitors p21 and p27 (Funk et al., 1997). Despite the ability of E7 to stimulate cell proliferation, during productive infection only a subset of cells in the parabasal layers are mitotically active. The expression of cyclin E is absolutely necessary for S-phase entry, and is expressed during natural infection as a result of E7 expression and disruption of the E2F/pRb complex. In differentiating epithelial cells, however, the high levels of cyclin-dependent kinase inhibitors (p21cip1 and p27kip1) can lead to the formation of inactive complexes that contain E7, cyclinE/cdk2 and either p21 or p27 (Noya et al., 2001). It appears that during natural infection, the ability of E7 to stimulate S-phase progression is limited to the subset of differentiated cells with low levels of p21/p27, or which express high enough levels of E7 to overcome the block to S-phase entry. The viral E6 protein complements the role of E7, and is thought to prevent the induction of apoptosis in response to unscheduled S-phase entry mediated by E7. Although the association of E6 with p53, and the inactivation of p53-mediated growth suppression and/or apoptosis has been well documented, E6 can also associate with other pro-apoptotic proteins including Bak (Thomas and Banks, 1998) and Bax (Li and Dou, 2000). As a consequence, the presence of E6 is considered a predisposing factor in the development of HPV-associated cancers, allowing the accumulation of chance errors in host cell DNA to go unchecked. The E6 protein of high-risk HPV types can also stimulate cell proliferation independently of E7 through its C-terminal PDZ-ligand domain (Thomas et al., 2002). E6-PDZ binding is sufficient to mediate suprabasal cell proliferation (Nguyen et al., 2003a,b) and may contribute to the development of metastatic tumours by disrupting normal cell adhesion. In addition to E6 and E7, it is thought that the other viral early proteins (i.e., E1, E2, E4 and E5) are expressed prior to the onset of genome amplification in order to ensure maintenance of the viral episome at low copy-number (Middleton et al., 2003).

3.4. Genome amplification

For the production of infectious virions, papillomaviruses must amplify their viral genomes and package them into infectious particles. For supergroup A, HPV types such as HPV16, HPV11 or HPV2, this occurs in the mid or upper epithelial layers following an increase in activity of the late (differentiation dependent) promoter. The late promoter resides within the E7 open reading frame and it is thought that its up-regulation leads to increased expression of proteins involved in viral DNA replication (i.e., E1, E2, E4 and E5) without directly affecting expression of the E6 and E7 proteins that are necessary for S-phase entry (Middleton et al., 2003). Amplification of viral genomes begins in a subset of cells in the proliferative compartment and requires expression of all viral early gene products including E4 (Peh et al., 2004) and E8 (Fehrmann et al., 2003; Genther et al., 2003), whose roles in replication are not yet fully understood. The binding of E2 to the HPV upstream regulatory region is necessary for viral DNA replication, and recruits the E1 DNA helicase to the viral origin of replication. The assembly of the E1/E2 initiation complex on the viral origin is analogous to the formation of the complex between cellular initiation proteins (cdc6 and MCMs) on cellular origins, and may allow the replication of viral genomes to proceed in the absence of cellular DNA synthesis. Throughout the virus life cycle, the relative levels of different viral proteins are controlled by promoter usage and by differential splice site selection, with an increase in the level of E1 and E2 allowing an increase in viral copy number in the upper epithelial layers (Ozbun and Meyers, 1998a). The molecular mechanisms that lead to activation of the late promoter and up-regulation of E1/E2 expression are not yet well understood, and it remains possible that this promoter is constitutively active at all stages during the productive cycle. Current models suggest that a modest increase in promoter activation during differentiation may lead to an increase in the level of E1 and E2 (and also E4 and E5), and a subsequent increase in genome copy number. The newly replicated genomes would serve as templates for the further expression of E1 and E2, which would facilitate additional amplification of viral genomes and in turn, further expression of the E1 and E2 replication proteins (Middleton et al., 2003).

3.5. Virus synthesis

Papillomaviruses encode two structural proteins that are expressed in the upper layers of infected tissue once viral genome amplification has been completed (Ozbun and Meyers, 1998b). L2 is a minor coat protein that like L1 is produced in a subset of the cells that express E4 (Doorbar et al., 1997). The major capsid protein (L1) is expressed after L2 allowing the assembly of infectious particles in the upper layers of the epithelium (Florin et al., 2002). Papillomavirus particles comprise an approximately 8000 base pair genome within a capsid that contains 360 copies of the L1 protein,

and probably 12 copies of L2, organized into a T2 capsomere icosohedral shell (Modis et al., 2002). The L2 protein accumulates at nuclear structures known as PML bodies during virus assembly (possibly through association with the transcription factor Daxx (Becker et al., 2004)) and recruits L1 to these domains. It has been suggested that PML bodies may be the sites of papillomavirus DNA replication (Day et al., 1998; Swindle et al., 1999), and that capsid proteins accumulate at these sites to facilitate packaging. Although virus like particles can assemble in the absence of L2, the L2 protein is thought to enhance packaging (Stauffer et al., 1998; Zhou et al., 1993) and infectivity (Roden et al., 2001). To be successful, the virus must eventually escape from the infected skin cell and survive extra-cellularly prior to re-infection. Papillomaviruses are non-lytic, and are not released until the infected cells reach the epithelial surface. Papillomaviruses are resistant to desiccation (Roden et al., 1997) and their extra-cellular survival may be enhanced if they are shed from the epithelial surface within a cornified squame (Bryan and Brown, 2001). The intracellular retention of papillomavirus antigens until the cell reaches the uppermost epithelial layers may compromise the immune detection of the virus, particularly as the virus also has molecular mechanisms that limit the presentation of viral epitopes to the immune system in the lower epithelial layers (Ashrafi et al., 2002; Marchetti et al., 2002; Matthews et al., 2003). Although the expression of viral proteins can inhibit expression of differentiation markers preventing the formation of normal cornified squames (Doorbar et al., 1997), it has also been suggested that the viral E4 protein may contribute directly to virus egress in the upper epithelial layer by disturbing keratin integrity (Doorbar et al., 1991; Wang et al., 2004) and by affecting the assembly of the cornified envelope (Bryan and Brown, 2000; Lehr et al., 2004).

4. Life cycle organization amongst HPVs of different

Although all papillomaviruses must follow the broad pattern of events described above in order to produce infectious virions, different strategies of productive infection are apparent between the different evolutionary groups. Human papillomaviruses from the B2 supergroup such as HPV4 for instance, do not contain the LXCXE motif necessary for pRB association (Munger et al., 2001) in their E7 protein, suggesting that at a molecular level they may operate differently from viruses of supergroup A, such as HPV2, which cause lesions at similar sites. Similarly, the E4 protein of HPV4 appears to lack the classical keratin binding motif that is present in HPV1 (E supergroup) and HPV2 (A supergroup) (Doorbar, 1996), despite infecting cutaneous epithelium, and sharing the same requirements as other HPV types regarding escape from the cornified squame. Comparative analysis of papillomaviruses of different types has shown the E1 and L1 regions to be the most highly conserved (de Villiers et al., 2004). It appears that these ORFs are fundamental for the survival of all papillomaviruses, and that they were probably present in the ancestor of modern papillomaviruses. Despite the diversity amongst papillomaviruses, it appears that viruses from related evolutionary groups share certain similarities. This can be illustrated by comparing papillomaviruses contained within the E supergroup (such as HPV 1) with those from the A group (such as HPV2), both of which cause verrucas (Fig. 2). In the former group, which includes canine oral papillomavirus (COPV), genome amplification begins as soon as cells leave the basal layer, without the intervening proliferative-phase characteristic of viruses such as HPV2 or HPV11 (Middleton et al., 2003). It has been speculated that these differences may reflect differences in transmission routes of the different HPV types and the need to produce the appropriate number of virus particles to allow infection without stimulating immunity against infection. It is equally possible that the co-evolution of papillomaviruses with their hosts has led to adoption of different general strategies to achieve the same goal.

5. Regression of lesions and virus latency

Although genome amplification and packaging is necessary for the formation of new virions, infection can have other outcomes. Experimental inoculation of rabbits with ROPV, or the inoculation of dogs with COPV generally leads to the development of lesions that can persist for weeks rather than years (Christensen et al., 2000; Nicholls et al., 2001). Lesions produced by ROPV and COPV resemble in many respects those produced by HPVs, and these viruses have been proposed as models to study mucosal HPV infection in humans. Between weeks 8 and 12, lymphocyte infiltration and lesion regression takes place, and by 16 weeks, the infected area has the appearance of uninfected epithelium (Nicholls et al., 2001). A similar pattern has been reported in cattle (Knowles et al., 1996) and may also occur in humans under some circumstances (Coleman et al., 1994). The importance of the immune system in controlling the spread of HPV-associated disease is well established, and patients with immune defects are particularly susceptible to infection, and can develop widespread lesions that are refractory to treatment. HPV infections are a particular problem in renal transplant and other immunosuppressed patients, in HIV infected individuals, and in some patients with genetic defects that affect immune cell function. The failure of patients suffering from Epidermodysplasia Verruciformis (Ramoz et al., 2002) or certain forms of severe combined immunodeficiency syndrome (Laffort et al., 2004) to control infection implicates a role for specific pathways of immune regulation. The inapparent infections associated with viruses of the B1 supergroup may resemble infections by other HPV types once they have been brought under control by the host immune system (Stern, 2005). The frequent detection of HPV16 DNA in cervical lesions in the absence of any obvious disease, may be explained by its

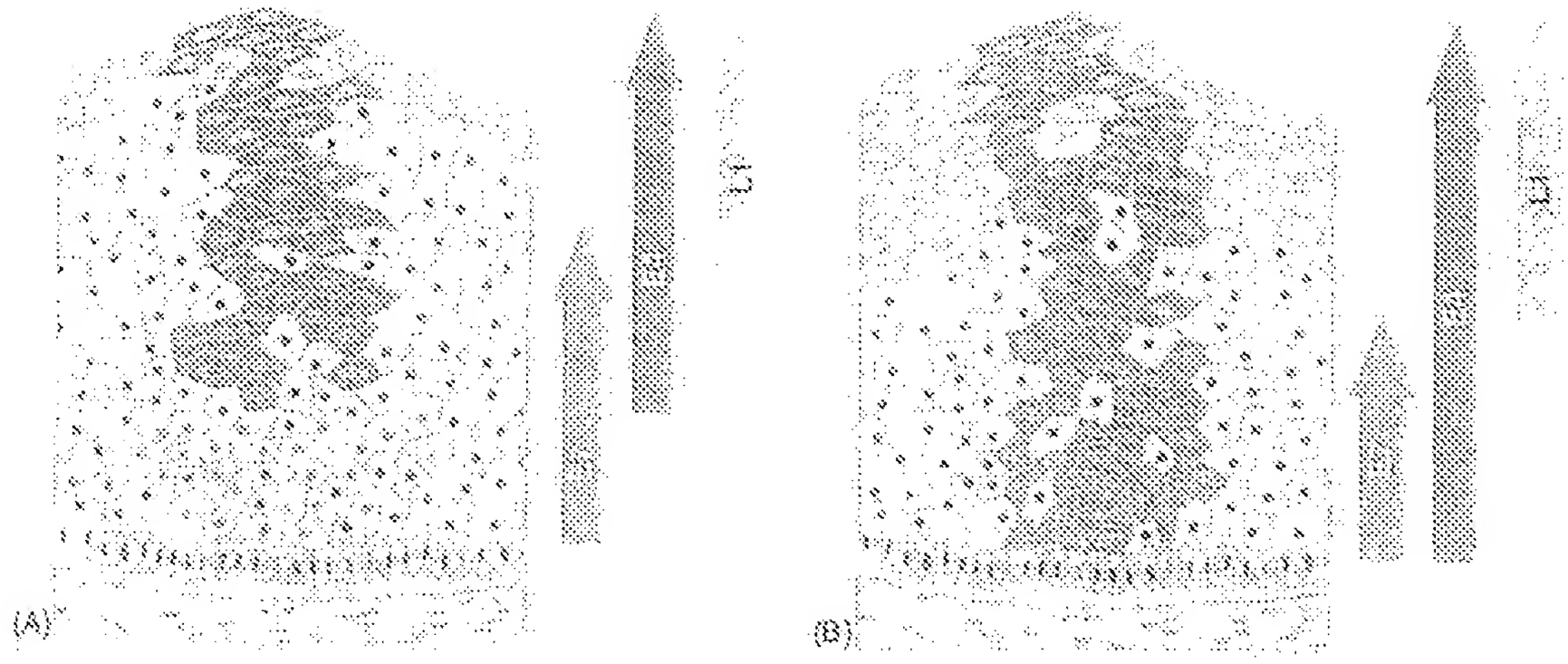

Fig. 2. Differences in life cycle organization amongst evolutionarily distinct HPV Types. When compared to HPVs from supergroup A such as HPV2 or HPV11 (left panel labelled A), those from supergroup E such as HPV1 or HPV63 (right panel labelled B), begin their productive cycle close to the basal layer. In lesions caused by these HPV types, there is no separate compartment where E7 can be detected in the absence of E4, and viral genome amplification begins in the parabasal cell layers (Peh et al., 2002).

presence in a latent state, with only very few cells (if any) able to support the productive cycle during epithelial cell differentiation. Following immune regression, papillomavirus DNA is thought to remain in the basal epithelial cells and to be reactivated when levels of immunosurveillance decline. The pattern of viral gene expression in basal cell layers during latent infection may be similar to the expression pattern in these layers during productive infection. It has been suggested that latent gene expression is restricted to E1 and E2 and that during this phase of the virus life cycle, the E6 and E7 genes are not required (Zhang et al., 1999).

6. Productive infection, abortive infection and HPV-associated cancers

In the absence of regression, lesions may persist, and may in some instances progress to cancer. A common characteristic of tumour viruses is their ability to cause tumours at sites where their productive life cycle cannot be completed. This general characteristic appears to hold true for papillomavirus-associated cancers, such as those caused by cottontail rabbit papillomavirus (CRPV) in domestic rabbits, and by BPV1 in horses (Campo, 2002). High-risk HPVs from supergroup A have been associated with human cervical cancers (Walboomers et al., 1999) whereas viruses from the B supergroup (particularly B1) have been implicated in the development of non-melanoma skin cancer (Harwood et al., 2004). When compared to the prevalence of HPV infections in the general population, the number of lesions that progress to cancer is very low. High-risk papillomaviruses from the A supergroup infect genital sites in men and women and cause flat lesions at cervical sites. In women, who do not successfully resolve their infection, such lesions can progress to cervical intraepithelial neoplasia grade 1 (CIN1), and may progress further to CIN2, CIN3 and cancer (Steenbergen, 2005; Gross and Barrasso, 1997; Peto et al., 2004). Low-grade cervical lesions (CIN1) resemble productive infections

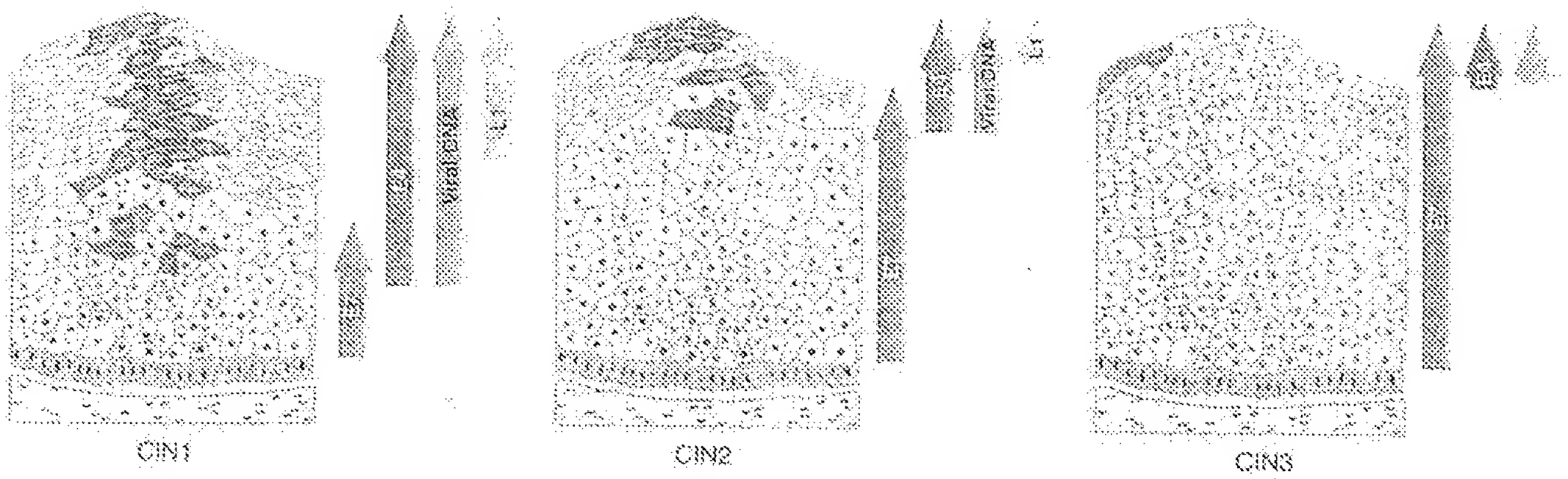

Fig. 3. Changes in the HPV16 life cycle during the development of cervical cancer. During progression from cervical intraepithelial neoplasia grade 1 (CIN1) to CIN3, normal regulation of the papillomavirus life cycle is lost. CIN1s generally resemble productive lesions caused by other supergroup A HPV types, and express virus coat proteins at the epithelial surface. In CIN2 and CIN3 lesions, the order of life cycle events is unchanged, but the extent of E7 expression is increased.

caused by related HPV types, while high-grade lesions such as CIN2 and CIN3 have a more extensive proliferative-phase, with the productive stages of the virus life cycle being supported only poorly (Middleton et al., 2003). The key event in the progression of productive lesions to high-grade neoplasia may result from a deregulation in the expression of the viral transforming proteins (E6 and E7), which leads to increased cell proliferation in the lower epithelial layers and an inability to repair secondary mutations in the host cell DNA (von Knebel Doeberitz, 2002). It is generally thought that the transformation zone is a particularly susceptible site for cervical cancer to develop. It appears that high-risk HPV types such as HPV16 cannot reliably complete their life cycle at this site, occasionally leading to abortive infection (Fig. 3). The progression from CIN3 to cancer usually occurs in lesions that contain integrated copies of the viral genome in which E7 expression is elevated. Thereafter, retention of the E6 and E7 genes and loss of the E2 and E4 genes, which can exert a negative effect on cell growth, usually accompanies the development of invasive cervical cancer. Although other HPV types such as those from the B1 supergroup are also associated with human cancers (i.e., non-melanoma skin cancer), in these cases, the integration of HPV sequences into host cell genome is not necessary, and viral sequences are not universally retained (Jablonska and Majewski, 1994; Harwood et al., 2004).

7. Life cycle organization amongst animal papillomaviruses

The general concepts that relate to the life cycles of human papillomaviruses appear to be applicable to the animal systems that are used to study infection (Peh et al., 2002). In many instances, animal papillomaviruses fall into evolutionary groups that contain no human members suggesting that they have been following an evolutionary path that is distinct from that followed by the HPVs (de Villiers et al., 2004). An exception to this are viruses from the B supergroup, which are widespread in animals (Antonsson and Hansson, 2002), and viruses from the E supergroup, which have been found in rabbits, cats and dogs. The medically important viruses from the A supergroup appear restricted to primates. With the exception of bovine papillomaviruses (BPV), the natural history of infection in animals has not been extensively studied. BPV1 and BPV2 are fibropapillomaviruses and produce lesions that have underlying dermal involvement. BPV1 causes cutaneous warts in its natural host, but induces fibroblastic tumours in horses. Although these viruses have been extensively studied because of their ability to transform cells in culture, they are evolutionarily distinct from the papillomaviruses that cause tumours in humans. The second important group of bovine papillomaviruses includes BPV4, which can induce gastrointestinal tumours that can progress to cancer in cattle that have bracken fern in their diet (Campo, 2002). As with other papillomavirus-induced cancers, it is thought that infection leads to an expansion in the number of dividing cells, and that in the presence of co-carcinogens (quercetin in bracken fern), the accumulation of secondary genetic changes can lead to cancer. In the case of human viruses from the B1 supergroup, the co-factor has been identified as UV light, whereas for HPV-associated cervical lesions, metabolites from smoking present an increased risk of cancer progression.

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